EMERGING ISSUES

Peer Review
Experimental Toxicology Division
NHEERL

Development of Methods for Analysis of Vinclozolin and its Metabolites in Biological Media

Adolfo Sierra-Santoyo[1], Hugh A. Barton[2] and Michael F. Hugues[2]

[1]National Research Council and [2]Pharmacokinetics Branch

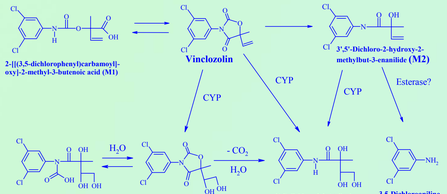
INTRODUCTION

The dicarboximide fungicide vinclozolin (V) is used on treatment of grains, grapes, fruits, ornamental plants and turfgrass (EPA, 1998; Szeto et al., 1989). As a result of its widespread use the analysis of V and other fungicides residues have been incorporated in the US Food and Drug Administration (FDA) Pesticide Residue Monitoring Program of food samples. Recent concern for the possible effects of endocrine-disrupting chemicals on humans and wildlife (Colborn et al., 1996) has resulted in considerable interest in environmental contaminants that affect aspects of reproduction and early development.

V is a proven endocrine disruptor causing anti-androgenic effects. Administration of V to pregnant rats at specific gestational stages resulted in a significant degree of morphological feminization and demasculinization of male offspring (Gray et al., 1994). Other studies indicated that these effects appeared to be predominantly due to antiandrogenic effects of M1 and M2 (Kelce et al., 1994; 1997). M1 and M2 bind to the mammalian androgen receptor (AR), and act as AR antagonists. They interfere with androgen-dependent gene expression *in vivo* and *in vitro* by inhibiting AR-binding to DNA (Gray et al., 1994; Monosson et al., 1999; Wolf et al., 2000).

A third metabolite, 3,5-dichloroaniline (M3) has been shown to be embryolethal, genotoxic, and teratogenic in animal studies (USEPA, 1987). Human exposure to this compound is considered a health hazard (Pothuluri et al., 1991).

Knowledge about the fate of V residues in animals and human is scarce. Due to the lower concentrations levels and the small sample amounts available, analytical procedures for the determination of V in human body fluids need to be improved and to be more powerful than those for food or workplace monitoring.



Proposed scheme of biotransformation of vinclozolin

OBJECTIVES

1. To optimize an HPLC method for the analysis of V and its metabolites.
2. To develop extraction methods of V and its metabolites from rat serum and tissues.
3. To evaluate the stability of V, M1 and M2 in different media.

Chromatographic Conditions

Solvents A: 0.05 M Phosphate buffer pH 3.3
B: Methanol:Acetonitrile (70:30)

Gradient B: 60 – 70%, 0 - 20 min.
70 – 75 %, 20 - 25 min.
60% 26-30 min

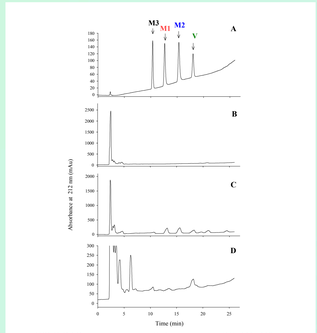
Column Nucleosil 100-5 C-18 AB, 5 µm 250X4.6 mm
Guard column C-18

HPLC Agilent Series 1100

DAD λ = 212, 219 and 280 nm
$\lambda_{ref.}$ = 550 nm

Result

1. In these chromatographic conditions there is an excellent resolution for V and its metabolites.
2. The most sensitive λ was 212 nm.
3. Detection limits for V, M1, M2 and M3 were from 0.5 to 2.2 µM.



HPLC-DAD chromatograms of: A, vinclozolin (V), M1, M2 and M3 dissolved in methanol; B, a serum sample of non treated rat; C, a serum sample of non treated rat spiked with 30 µg/ml of V and each metabolite; and D, serum from a rat administered V at a dose of 100 mg/kg.

Extraction Method

To 100 µl of rat serum, liver homogenate, 0.01 M PB pH 7.4 or incubation medium for enzyme assays aliquot 100 µl were added 400 µl 0.1 M PB pH 3.3. All samples were spiked with 10 µg/ml of V and its metabolites. V and its analytes were extracted with 5 ml acetonitrile. Samples were vortexed for 1 min and centrifuged at 3500 rpm for 10 min at 4ºC. Supernatant was dried using anhydrous sodium sulphate and concentrated under N_2. Extracts were redissolved in 200 µl methanol before HPLC or stored at 4°C until analysis.

Result

1. Extraction in acid conditions is useful for V, M1 and M2 analysis in PB, rat serum and liver homogenate, and incubation medium for enzyme assays.
2. M3 analysis after extraction in acid conditions may be underestimated, therefore, other conditions could be developed.

Recovery (%) of V and its metabolites in spiked rat serum samples using different solvents for extraction

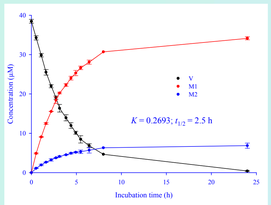
Solvent	Medium	M3	M1	M2	V
Ethyl acetate	PB pH 7.4	30.8	87.1	77.9	76.4
	Serum	32.9	92.7	83.4	80.6
Methanol	PB pH 7.4	1.9	85.3	81.2	97.0
	Serum	9.8	98.5	89.0	117.0
Acetonitrile	PB pH 7.4	3.3	90.9	85.5	90.0
	Serum	22.6	88.5	81.2	91.0

Stability Assays

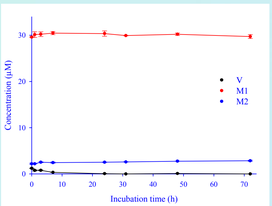
V or its metabolites at a concentration of 10 µg/ml were incubated in phosphate buffer pH 7.4 or serum of Long Evans male rats of 70 days old at 37 °C. Aliquots of 100 µl were collected at different times for metabolite analysis. Non linear regression analysis was performed using SigmaPlot software.

Result

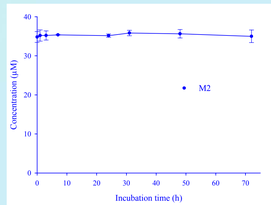
1. V is very susceptible to chemical hydrolysis in rat serum and phosphate buffer pH 7.4 incubated at 37 °C.
2. a) V is hydrolyzed to M1 and M2.
   b) M1 is transformed to V and M2.
3. M1 represents around 70% of the relative concentration of final product of V hydrolysis.
4. V $t_{1/2}$: In serum, 0.5 h; in phosphate buffer pH 7.4, 2.5-2.9 h.



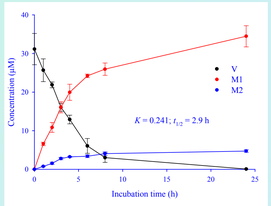
Vinclozolin hydrolysis kinetics in Phosphate Buffer pH 7.4 at 37°C. Vinclozolin and its metabolites were analyzed by HPLC-DAD without extraction process. Values are mean ± SD (n=2).



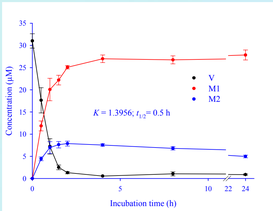
Stability of M1 in Phosphate Buffer pH 7.4 at 37°C. Metabolites were analyzed by HPLC-DAD without extraction process. Values are mean ± SD (n=2).



Stability of M2 in Phosphate Buffer pH 7.4 at 37°C. Metabolites were analyzed by HPLC-DAD without extraction process. Values are mean ± SD (n=2).



Vinclozolin hydrolysis kinetics in Phosphate Buffer pH 7.4 at 37°C. Vinclozolin and its metabolites were extracted in acetonitrile and analyzed by HPLC-DAD. Values are mean ± SD (n=3).



Vinclozolin hydrolysis kinetics in rat serum at 37°C. Vinclozolin and its metabolites were extracted in acetonitrile and analyzed by HPLC-DAD. Values are mean ± SD (n=3).

CONCLUSIONS

1. The chromatographic conditions developed in this study allow quantitative analysis of V and its metabolites in different biological samples such as rat serum, tissue homogenates and incubation media from enzyme assays.
2. Hydrolysis of V and its metabolites in serum and phosphate buffer pH 7.4 is mainly non-enzymatic. Serum proteins may play an important role for the stability and bioavailability of V and its metabolites.
3. M1 is the main product of V hydrolysis and represents about 70% of total products.

IMPACT

1. This method may be used for the analysis of V and its metabolites in pharmacokinetic studies that can lead to physiologically-based pharmacokinetic model development for the anti-androgenic toxicant V.
2. The results from this project will help reduce the uncertainity of the risk assessment of V and potentially other dicarboximide fungicides.

SOLVING AGENCY PROBLEMS